SUPPLEMENTARY INFORMATION

In vitro fermentation test bed for evaluation of engineered probiotics in polymicrobial communities

Authors

Steven Arcidiacono[1], Amy M. Breedon[2], Michael S. Goodson[2], Laurel A. Doherty[1], Wanda Lyon[2], Grace Jimenez[2], Ida G. Pantoja-Feliciano[1], Jason W. Soares[1*]

*Corresponding author
jason.w.soares.civ@mail.mil

Authors' affiliations

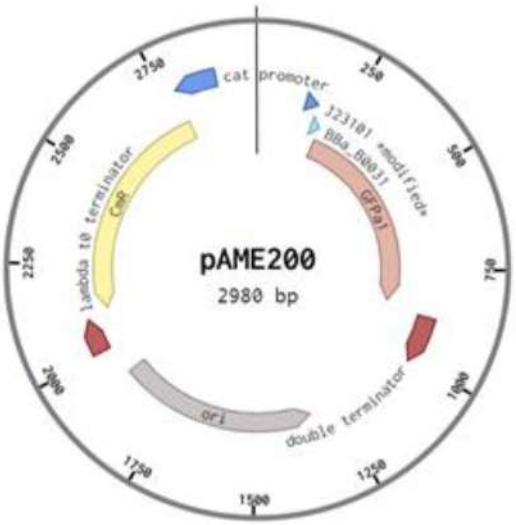
[1]Soldier Effectiveness Directorate, DEVCOM Soldier Center, Natick, MA 01760
[2]711th HPW, Air Force Research Laboratory, Dayton, OH 45433

Abbreviations

E. coli Nissle (EcN); *E. coli* Nissle wild type (EcNWT); *E. coli* Nissle engineered for GFPa1 synthesis (EcN(cGFP))

Table of content

Figure S1. Plasmid construction and strain engineering of *E. coli* Nissle 1917 for constitutive production of the fluorescent protein GFPa1.



DNA encoding the green fluorescent protein reporter GFPa1 was cloned downstream of a constitutive promoter and expressed from a plasmid containing chloramphenicol resistance. GFPa1 (Bomati (2009) BMC Evol Biol), along with ribosome binding site and terminator regions, was amplified from pAMP (Goodson et al (2017) ACS Synth Biol) using primers AME1000 (TCTAGAGTCACACAGGAAAC) and AME1001 (TATAAACGCAGAAAGGCCC). The PCR product was reamplified with primers AME1007 (GCTTTCGCTAAGGATGATTTCTGGAATTCGGATCCTTTACATTTAGCTCAGTCCTAG GTATTATGCTAGCTCTAGAGTCACACAGGAAAC) and AME1002 (TTGCCCGTTTTTTTGCCGGACTGCAGCGGCCGCTATAAACGCAGAAAGGCCC) to add promoter (underlined; two-point mutation version of BioBrick promoter BBa_J23101 from the Anderson Promoter Collection; Registry of Standard Biological Parts http://partsregistry.org) and overlap regions and was cloned into plasmid J64100 at *Eco*RI/*Not*I using Gibson Assembly to create pAME200. The plasmid was verified via sequencing using primers AME1012 (TGCCACCTGACGTCTAAG) and AME1013 (GACCGAGCGCAGCGAG). pAME200 was transformed into *E. coli* Nissle 1917 using standard heat shock technique and designated EcN(cGFP). J64100 was a gift from Prof. Chris Voigt (MIT). *E. coli* Nissle 1917 was a gift from Dr. Scott Walper (Naval Research Laboratory).

Table S1. Complex Colonic Medium (CCM) recipe (based on Macfarlane (1998) Microb Ecol). CCM contains soluble and insoluble components and designed to simulate the gut nutritional environment to completely support microbe growth.

	Ingredient	per liter
Soluble components	Tween 80	1 ml
	Peptone	5 g
	Yeast extract	4.5 g
	Tryptone	5 g
	NaCl	4.5 g
	KCl	4.5 g
	$NaHCO_3$	1.5 g
	$MgSO_4$-$7H_2O$	1.25 g
	KH_2PO_4	0.5 g
	$FeSO_4$-$7H_2O$	0.005 g
	$CaCl_2$-$2H_2O$	0.02 g
	Cysteine-HCl	0.8 g
	Hemin (98%)	0.05 g
	Resazurin	0.01g
Insoluble components	Potato starch	5 g
	Pectin	3 g
	Guar gum	2 g
	Xylan (oat spelt)	2 g
	Arabinogalactan	2 g
	Inulin (dahlia)	1 g
	Casein (bovine milk)	3 g
	Bile salts #3	0.4 g
	Mucin (porcine type III)*	4 g
	pH to 7.0 with 1M NaOH	

* Note: Add mucin last and stir for 2-3 hours to ensure a uniform resuspension.

Table S2. Primers sequences used for qPCR.

Primer designation	Primer sequence (5'to3')	uM primer	Target
Mut7f*	GACCAAGCGATAACCGGATG	5 uM	MUT2 plasmid
Mut8r*	GTGAGATGATGGCCACGATT	5 uM	
GFPa1-f	GCAAGCTGTCTACCGAGTTTA	5 uM	pAME200 GFPa1 plasmid
GFPa1-r	AAGGGCTTGTCGAAGATGTAG	5 uM	

Primers targeting the MUT2 plasmid that is naturally occurring in *E. coli* Nissle were used to determine EcN(cGFP) and EcNWT persistence. Target efficiency; E = 80 to 100%. qPCR amplification of the off-target commensal organisms was $\leq$0.1%.

* Blum-Oehler (2003) Res Microbiol.

Figure S2: Bacteria concentration calculation determining log CFU/ml culture organism persistence using sample Ct values from qPCR.

Calculating sample log CFU/ml culture

After determining the sample log CFU value from the standard curve, convert to log CFU/ml culture

For samples, assume:

- Volume culture = 5 ml
- Volume DNA solution after extraction = 100 ul
- Volume DNA solution in qPCR reaction = 2 ul

Calculations:

1. Use sample Ct to determine log CFU from standard curve
2. Convert log CFU to linear number (Excel POWER function) = CFU in qPCR reaction
3. Convert CFU in qPCR reaction to log CFU/ml culture
   - *Example*: If a 2 ul qPCR reaction = 7500 CFU:

$$\frac{\text{7500 CFU}}{\text{2 ul reaction}} \times \frac{\text{100 ul extract}}{\text{5 ml culture}} = \text{75000 CFU/ml culture}$$

LOG(75000 CFU/ml culture) = 3.8 log CFU/ml culture

NOTE: if extraction solution needs to be diluted (e.g., due to high DNA concentration, presence of inhibitors), account for dilution factor in calculation

Figure S3. Immunodetection of GFPa1 protein expression.

Cell lysates were run on NuPAGE 12% Bis-Tris gel (Invitrogen, Carlsbad, CA) gels in MOPS buffer according to the manufacturer's protocol. Protein was transferred onto NitroPlus nitrocellulose (Micron Separations, Inc., Westborough, MA) with carbonate transfer buffer (g/L: 0.84g $HaHCO_3$, 0.318g Na_2CO_3, 100 ml methanol) for 2 h at 80 mA using IMM-1 Semi-dry blotting system (WEP Co, Seattle, WA). All washes, antibody incubations and detection were done with gentle mixing. Nitrocellulose membrane was blocked for 1 h with 5% Bovine Serum Albumin in Tris Buffered Saline Tween (TBST), g/L: Tris base, 3.3; NaCl, 8.8; KCl, 0.2, pH to 7.4 with concentrated HCl, Tween 20 added to 0.1% (v/v), then washed with TBST for 5 min. Immunodetection of GFPa1 was achieved with a 1:1000 dilution (in blocking buffer) of rabbit anti-mNeonGreen antibody (Cell Signaling Technologies, Danvers, MA) incubated 4°C overnight. After washing 3x with TBST for 5 min, a 1:1000 dilution (in blocking buffer) of anti-rabbit antibody conjugated to alkaline phosphatase, washing 2x with TBST for 5 min and 1x TBS for 5 min. Detection was done using an Alkaline Phosphatase (AP) Conjugate Substrate Kit (BioRad, Hercules, CA) in 100 mM Tris-Cl, pH 9.5.

Figure S3A. Western blot of EcN(cGFP) 24 h culture lysates. M1. Novex Sharp marker, pre-Western; 1. EcN(cGFP) mono; 2. EcN(cGFP) 4-member; 3. EcN(cGFP) 6-member; 4. EcN(cGFP) 8-member; M2. Novex Sharp marker, post-Western. M1 Novex Sharp lane cut from NC membrane post-blot and dried. Remaining NC brought through Western protocol. Western blot analysis using mNeonGreen antibody showed increased production of GFPa1 in the 8-way (and to a lesser extent the 6-way) fermentations, confirming the fluorescent reporter function results. mNeonGreen antibody was used since there is no commercially available GFPa1 antibody.

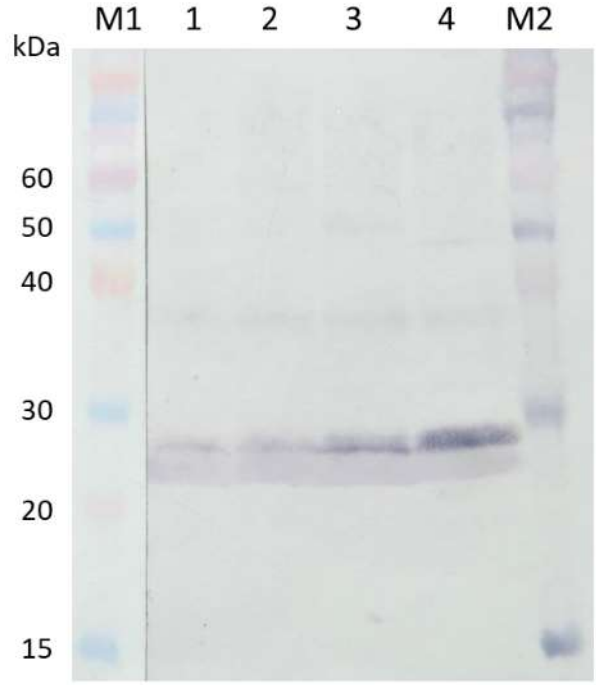


Figure S3B. Protein sequence alignments using Clustal Omega (UniProt.org).
Symbol key: * = fully conserved residue; : = strongly similar residue; . = weakly similar residue. mNeonGreen was found to be a suitable antibody for GFPa1 protein detection. GFPa1 is found in *Branchiostoma floridae* (Bomati 2009) a genus making a number of fluorescent proteins, including mNeonGreen in *B. lanceolatum* (Shaner (2013) Nat Meth). The sequence homology of mNeonGreen to that of GFPa1 is 49.2%. Antibodies to the traditional *Aequorea victoria* GFP do not react with GFPa1, as there is only a 20.9% homology between their sequences.

GFPa1 vs mNeonGreen.

```
GFPa1      ----------MPLPATHDIHLHGSINGHEFDMVGGGKGDPNAGSLVTTAKSTKGALKFSP 50
mNeonGreen MVSKGEEDNMASLPATHELHIFGSINGVDFDMVGQGTGNPNDGYEELNLKSTKGDLQFSP 60
                     *****::*:.***** :***** *.*:** *    . ***** *:***

GFPa1      YLMIPHLgygyyqylpypdgpSPFQVSMLEGSGYAVYRVFDFEDGGKLSTEFKYSYEGSH 110
mNeonGreen WILVPHIGYGFHQYLPYPDGMSPFQAAMVDGSGYQVHRTMQFEDGASLTVNYRYTYEGSH 120
           ::::**:***::******** ****.:*::**** *:*.::****..*:.:::*:*****

GFPa1      IKADMKLMGSGFPDDGPVMTSQIVDQDGCVSKKTYLNNNTIVDSFDWSYNLQNGKRYRAR 170
mNeonGreen IKGEAQVKGTGFPADGPVMTNSLTAADWCRSKKTYPNDKTIISTFKWSYTTGNGKRYRST 180
           **.: :: *:*** ******..:.  * * ***** *::**:.:*.***.  ******:

GFPa1      VSSHYIFDKPFSADLMKKQPVFVYRKCHVKATKTEVTLDEREKAFYELA------- 219
mNeonGreen ARTTYTFAKPMAANYLKNQPMYVFRKTELKHSKTELNFKEWQKAFTDVMGMDELYK 236
           . : * * **::*: :*:**::*:** .:* :***:.:.* :*** ::
```

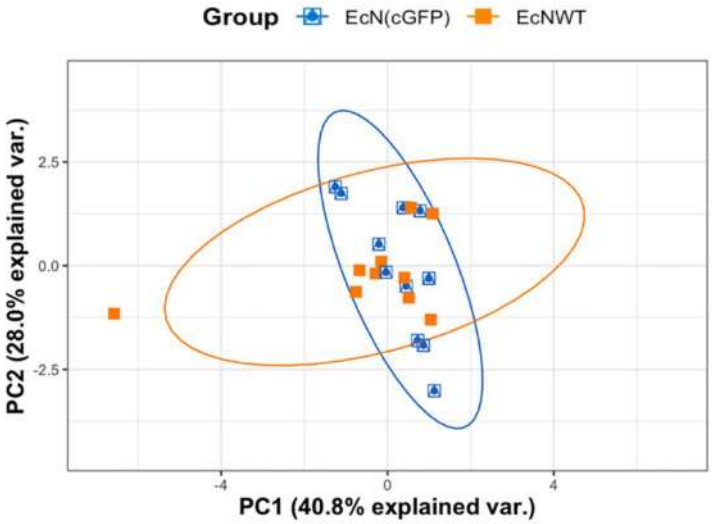
Sequence similarity 49.2%

GFPa1 vs *Aequorea victoria* GFP.

```
GFPa1        -------MPLPATHDIHLHGSINGHEFDMVGGGKGDPNAGSLVTTAKSTKGALKFSPYLM 53
Aequorea_GFP MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTL 60
                    :   .   :.*.*.:***:*.: * *:** . *.*. .  .*.* * .    :

GFPa1        IPHLgygyyqylpypdgpSPFQV-SMLEGSGYAVYRVFDFEDGGKLSTEFKYSYEGSHIK 112
Aequorea_GFP VTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLV 120
             :  :.**   :  ***  . .:. .    .**.  *.: *:*.*: .*. : .:**. :

GFPa1        ADMKLMGSGFPDDGPVMTSQIVDQDGCVSKKTYLNN----NTIVDSFDWSYNLQNGKRYR 168
Aequorea_GFP NRIELKGIDFKEDGNILGHKLEYNYN--SHNVYIMADKQKNGIKVNFKIRHNIEDGS--- 175
               ::* * .* :** ::  ::  : .  *::.*:      * *  .*.  :*:::*.

GFPa1        ARVSSHYIFDKPFSADLMKKQPVFVYRKCHVKATKTEVTLDEREK----AFYELA----- 219
Aequorea_GFP VQLADHYQQNTPIGD-----GPVLLPD-NHYLSTQSALSKDPNEKRDHMVLLEFVTAAGI 229
             .:::.**  :.*:.       **::    *  :*:: :: * .**    .: *:.

GFPa1        ---------
Aequorea_GFP THGMDELYK 238
```

Sequence similarity 20.9%

Fig. S4. PCA of overall community dynamics comparing EcN(cGFP) to EcNWT. No distinctive clustering is observed, revealing no significant difference and that engineering EcN does not affect community dynamics.



References

Bomati EK, Manning G, Deheyn DD (2009) Amphioxus encodes the largest known family of green fluorescent proteins, which have diversified into distinct functional classes. BMC Evol Biol 9: 77.

Goodson MS, Bennett AC, Jennewine BR, Briskin E, Harbaugh SV, Kelley-Loughnane N (2017) Amplifying riboswitch signal output using cellular wiring. ACS Synth Biol 6: 1440-1444.

Registry of Standard Biological Parts. http://partsregistry.org

Blum-Oehler G, Oswald S, Eiteljorge K, Sonnenborn U, Schulz J, Kruis W, Hacker J (2003) Development of strain-specific PCR reactions for the detection of the probiotic *Escherichia coli* strain Nissle 1917 in fecal samples. Res Microbiol 154: 59–66.

Macfarlane GT, Macfarlane S, Gibson GR (1998) Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. Microb Ecol 35: 180-187.

Shaner NC, Lambert GG, Chammas A, Ni Y, Cranfill PJ, Baird MA, Sell BR, Allen JR, Day RN, Isrealsson M, Davidson MW, Wand J (2013) A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum. Nat Meth 10: 407-409.

UniProt Clustal Omega. https://www.uniprot.org/align/